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Review

The regulation of cell proliferation by the papillomavirus early proteins

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Abstract. The human papillomavirus (HPV) E6 and E7 oncogenes have direct effects on host cell proliferation. The viral E2 protein regulates transcription of E6 and E7 and thereby has an indirect effect on cell proliferation. In HPV-induced tumours, misappropriate random integration of the viral genome into the host chromosome often leads to disruption of the E2 gene and the loss of E2 expression. This results in cessation of the virus life cycle and the deregulation of E6 and E7 and is an important step in tumourigenesis. However, prior to these integration events, E2 can interact directly with the E6 and E7 proteins and modulate their activities. E2 also interacts with a variety of host proteins, including the p53 tumour suppressor protein. Here we outline evidence that suggests a role for E2 in the regulation of cell proliferation, and we discuss the importance of this regulation in viral infection and cervical tumourigenesis.

Keywords. Papillomavirus, cell proliferation, oncogene, transcription, cancer.

Introduction

Papillomaviruses infect epithelial cells and induce the formation of hyperproliferative lesions known as warts. Although most papillomavirus infections produce benign lesions, some virus types are associated with the formation of malignant tumours, most notably perhaps cervical cancer [1, 2]. This review will describe the roles of the human papillomavirus (HPV) early proteins during viral infection and cervical tumourigenesis, with a particular focus on the papillomavirus E2 protein and its influence on host cell proliferation and cell death.

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Human papillomaviruses

Cervical cancer is a major cause of cancer-related death in women worldwide, with around 500,000 new cases and 300,000 deaths in 2002 [3]. For almost three decades, evidence has been accumulating to implicate HPV as the causative agent of this disease [4]. Over 100 different types of HPV have been identified on the basis of their DNA sequence [5]. HPV types such as HPV 16 and HPV 18, that are associated with cervical cancer and other invasive tumours, are known as 'high oncogenic risk' types. In contrast, 'low oncogenic risk' types, such as HPV 6 and HPV 11, are found primarily in benign warts [6, 7]. More than 99 % of cervical cancers contain DNA from at least one high-risk HPV type, and approximately 70 % contain HPV 16 or 18 [1]. HPV vaccines should offer protection against virus-induced tumours and two vaccines have recently

become available. A bivalent HPV 16/18 vaccine is produced by GSK and a quadrivalent HPV 6/11/16/18 vaccine is produced by Merck [8, 9]. HPVs are also associated with a range of other tumours, including cancer of the vulva, oral cancer and skin cancer [10–13]. However, we will concentrate here on the role of HPV in cervical tumourigenesis.

HPVs are small, non-enveloped DNA viruses with a circular genome of around 8kb [14]. The viral genome can be divided into three regions (Fig. 1). The upstream regulatory region (URR), also known as the long control region (LCR), is largely responsible in determining the host range and tissue tropism of each HPV type and regulates viral gene expression after infection [14]. This region contains the viral origin of replication, four binding sites for the viral E2 protein and numerous binding sites for cellular transcription factors [15, 16]. The rest of the genome comprises eight open reading frames (ORF) divided between the early region, that encodes the E1, E2, E4, E5, E6 and E7 proteins and the late region, that encodes the L1 and L2 proteins (Table 1). The LCR contains an early promoter that expresses both the early and late genes, whereas a late promoter located within the E7 ORF predominantly expresses the late genes [17]. However, the situation is not clear-cut since some early genes can be expressed from the late promoter and vice versa. In addition, both promoters give rise to transcripts that undergo alternative splicing.

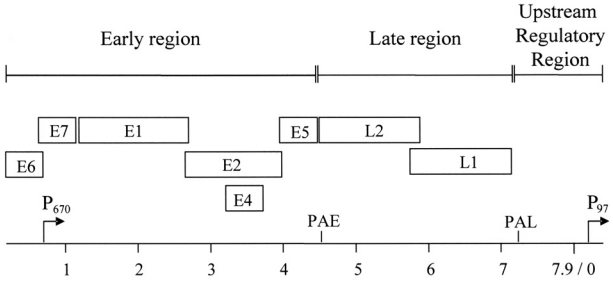

Figure 1. The HPV genome. The bottom line shows a linear representation of the circular 7.9kb HPV 16 genome. The bent arrows represent the early (p97) and late transcription start sites (p670). Early and late polyadenylation signals are indicated by PAE and PAL, respectively. The viral ORFs are indicated (middle section) as well as the early, late and upstream regulatory regions (top line). Splice variants such as E1 E4 and E8 E2C are not shown.

The early gene products are primarily involved in viral DNA replication and the subversion of host cell regulatory pathways. The E1 protein is required for HPV replication and is highly conserved among all HPV types [18, 19]. E1 is expressed throughout the HPV life cycle but the highest levels of E1 expression occur during the vegetative stage when the late promoter is active [20, 21]. E1 has helicase activity

Table 1. The HPV proteins and their functions.

Protein	Function
E1	Replication
E2	Transcription, replication
E4	Disruption of cytokeratin networks/cell growth arrest
E5	Transformation
E6	Transformation (binds to p53 amongst other proteins)
E7	Transformation (to pRb amongst other proteins)
L1	Major capsid protein
L2	Minor capsid protein

and binds to the HPV origin of replication prior to the initiation of DNA synthesis [22]. However, E1 alone does not bind to the origin with high affinity [23]. The E2 protein is a sequence-specific DNA binding protein with important functions in both viral replication and transcription [24]. E2 binds to DNA sequences within the LCR, and via a protein-protein interaction recruits E1 to the HPV origin [23, 25, 26]. E2 also plays important roles in the regulation of HPV transcription [24, 27–29] and in the segregation of HPV genomes between daughter cells after cell division [30–33].

The E6 and E7 proteins have transforming properties and are important during viral replication and in tumourigenesis. The E6 proteins from "high oncogenic risk" HPV types bind to the cellular tumour suppressor protein p53 and facilitate the turnover of p53 by forming a complex with the cellular ubiquitin ligase E6AP [34–36]. E6 can also block the transcriptional activity of p53 by inhibiting the transcription coactivator p300 [37]. E7 binds to members of the retinoblastoma (Rb) tumour suppressor family to facilitate progression in the cell cycle, from G_1 to S phase [38]. E7 also induces abnormal centrosome number and genomic instability and can induce chromosome misalignment and lagging chromosomes during mitosis [39, 40]. E6 and E7 possess immortalizing and transforming properties [41]. Immortalization occurs when cells become capable of continuous proliferation and proliferate indefinitely. In contrast, transformation describes the conversion of normal cells to abnormal cells in terms of their phenotype. Transformed cells typically exhibit anchorage-independent growth, altered cell morphology, loss of contact inhibition, increased proliferation and tumourigenicity in nude mice. The E5 protein has an additive effect on the transforming properties of E6 and E7 [42, 43]. E5 can be considered as an oncogene since it has the ability to transform mouse fibroblasts and keratinocytes in conjunction with E7 [44, 45]. This property of E5 is probably related to the protein's

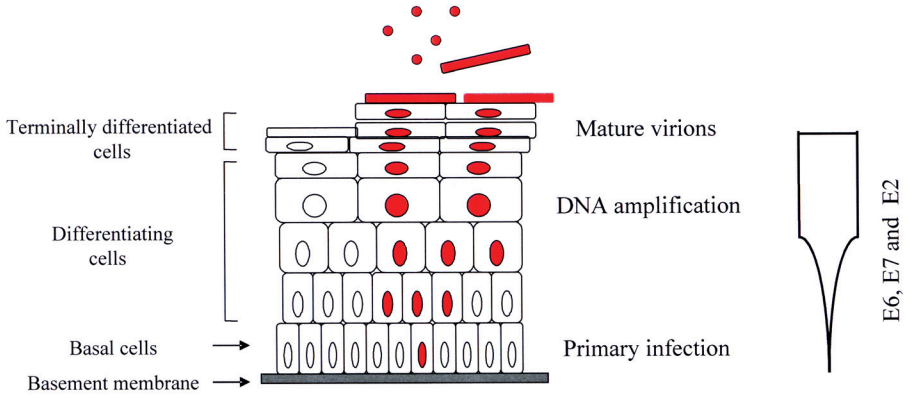

Figure 2. HPV infection. A schematic representation of a section through HPV-infected epithelium. Basal cells move up through the epithelium and undergo terminal differentiation. Infected cells are in red. HPV genome amplification and virus production occur in the upper layers.

ability to modulate cell signalling most likely through association with the vacuolar proton ATPase and the consequent delay of endosomal acidification. However, the effects of loss of E5 in the context of whole HPV 16 and HPV 31 genomes are not pronounced, again suggesting that this protein plays an additive rather than a major role in transformation [46, 47]. The precise role of E4 protein also remains unclear. However, E4 is associated with the collapse of cellular cytokeratin networks and this could facilitate viral release [48–50]. As in the case of E5, E4 appears to play a relatively subtle role in the viral life cycle [51–53]. Since E4 accumulates at late stages of infection, it is more accurately described as a late protein. The viral late region comprises two ORFs which encode the major (L1) and minor (L2) capsid proteins.

The HPV life cycle

The life cycle of HPV is thought to begin with the infection of epithelial stem cells (for a comprehensive review see [54]). In normal human epithelia, there are thought to be two types of keratinocytes; stem cells and transit amplifying cells. Stem cells are attached to the basement membrane and retain a high capacity for self-renewal. After the division of basal cells, daughter cells that will become transit amplifying cells migrate upwards and undergo terminal differentiation. Shortly after leaving the basement membrane, these normal cells exit the cell cycle and start to synthesize high molecular weight keratins that accumulate in the stratum granulosum and in the highest strata of the epithelium. These cells eventually form keratin-filled sacs in cutaneous epidermis.

HPV infection is thought to require a break in the stratified epithelium, which may occur through microlesions that expose the basal layer to viral entry. There appears to be redundancy in the receptors that allow attachment of the virus to the cell and/or different cell receptors for different viral types. Some studies have suggested that heparin sulphate proteoglycans play a role in the virus entry [55, 56], while other work has shown that entry occurs through the endocytosis of clathrin-coated vesicles [57, 58]. After virus entry, the virus maintains its genome at a low copy number and this requires the expression of E1 and E2. These proteins bind to the viral origin of replication and recruit cellular DNA polymerases and other proteins necessary for DNA replication [59]. The viral genome is thought to be maintained at 10–200 copies per cell in the basal layer throughout infection. However, this copy number is dramatically increased as the infected cells move into the upper layers of the epithelium (Fig. 2). During this amplification phase, the genomes are packaged into infectious virions before release. The infected cells become morphologically distinct koilocytes at this stage, with an enlarged nucleus and often multiple nuclei per cell. The activation of the late differentiation-dependent promoter leads to increased expression of the E1, E4 and E5 proteins [52, 53]. L1 and L2 are also expressed in the upper layer of the epithelium. The HPV virions are icosahedral in structure and are composed of 360 L1 proteins assembled into 72 pentameric structures [60]. L2 interacts with L1 and facilitates the assembly of virions [61]. The capsids undergo a maturation process triggered by the proteolytic processing of virion components before release from the cell. The virus that is shed can reinfect the basal epithelium or spread to new hosts. L1 can self-assemble into 72-pentamer virus-like particles (VLP) that resemble native papillomavirus virions [62] and can be used in vaccines [63].

HPV and cell proliferation

Several HPV proteins have either direct or indirect effects on cell proliferation (summarised in Fig. 3**).** The HPV E6 and E7 oncoproteins act to increase the proliferation of HPV-infected cells in the epithelium.

The activity of these proteins subverts the normal terminal differentiation process, resulting in increasing numbers of infected cells that will eventually produce infectious virions. Both E6 and E7 stimulate cell cycle progression and both of these proteins associate with regulators of the cell cycle [34, 64]. The best known function of E6 is its ability to bind to the p53 tumour suppressor protein in conjunction with the cellular ubiquitin ligase E6AP and target p53 for degradation [35, 65]. This impairs the normal cellular response to cell stress and DNA damage and allows infected cells to proliferate as a consequence of E7 expression. However, this is not the only function of E6 that influences cell proliferation and indeed the E6 proteins from HPV types associated with skin cancer do not interact with p53 but retain the ability to transform cells [66]. The association of E7 with members of the pocket protein family of cell cycle regulators has also been well characterized. pRb is a negative regulator of the cell cycle that controls S-phase entry by associating with members of the E2F family of DNA-binding transcription factors; E7 disrupts this function by binding to pRb and displacing E2F proteins [64]. Cells therefore enter S-phase and activate the cellular replication factors required for viral replication. E7 also targets pRb for proteolytic degradation by the ubiquitin proteasome pathway through the 26S proteasome [67–69]. In addition, E6 and E7 bind to a variety of other cellular proteins and the consequences of some of these interactions will be discussed in detail below. The HPV E5 protein also contributes to the increased proliferation of HPV-infected cells, although the mechanisms E5 uses to bring this about are less well understood. One possibility is that E5 inhibits apoptosis [70]. Conversely, the E1 E4 protein can induce growth arrest and this might be important to allow virus production or release at late stages of the viral life cycle [71]. Similarly, the E2 protein can induce growth arrest, cell senescence and apoptotic cell death. E2 can bring about these effects directly, by interacting with cellular proteins, or indirectly via its effects on the expression of E6 and E7 and the other viral proteins [72–74]. Furthermore, E2 is capable of interacting directly with both E6 and E7, leading to the modulation of their functions [75, 76].

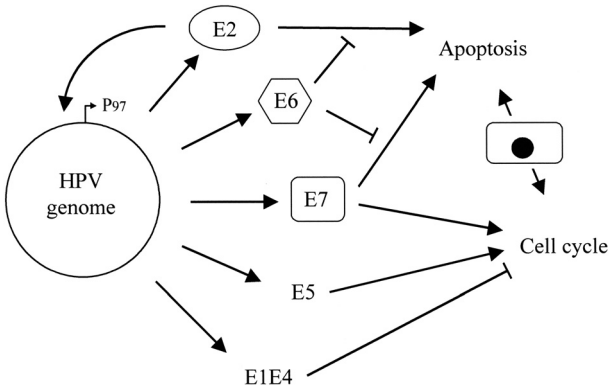

Figure 3. HPV proteins alter cell proliferation. The HPV genome produces several proteins that influence cell cycle progression. E6 can inhibit E7- and E2-induced apoptosis.

Tumourigenesis

In HPV transformed cells, the normally episomal HPV genome often becomes integrated into the host genome [77–79]. These random integration events leave the E6 and E7 oncogenes intact and they continue to be expressed. Interventions that inhibit the expression of E6 and E7 can reverse the transformed phenotype in these cells [72, 80–82]. In contrast, the E5 protein is not usually expressed in HPV transformed cells. These observations underline the importance of E6 and E7 in tumourigenesis. It is important to note that integration is not a normal part of the HPV life cycle and in fact, integration makes it impossible for the virus to complete its life cycle. There is evidence to suggest that episomal and integrated HPV genomes co-exist during the early phase of tumourigenesis [83]. At later stages the episomal forms become less common; however there is continued and even increased expression of E6 and E7. This is due in part at least to the increased stability of mRNA transcripts from the integrated genomes [84]. Cells with integrated HPV genomes show increased proliferation and presumably form a pool of immortalized cells within which further mutations can arise leading to full transformation and the formation of carcinomas. In addition, the E6 and E7 proteins promote genetic instability which will also lead to the emergence of carcinomas [85].

The number of integrated HPV genomes varies from tumour to tumour although not all of the integrated genomes are actively transcribed [86]. Integration occurs at different sites within the host genome in different carcinomas, but there does seem to be preferential integration at host chromosomal fragile sites [79, 87]. Disruption of the viral genome often occurs within the E2 gene. This leads to the loss of E2 expression and as described in detail below, the loss of regulation of E6 and E7 expression by E2. Integration can also disrupt the HPV genome within the E1 gene [79]. This also leads to the loss of E2 expression and consequently the loss of normal control over E6 and E7. The loss of the E2 protein thus plays a central role in HPV-induced tumourigenesis. However, as mentioned above, the E2 protein can also influence cell

proliferation by interacting with the E6 and E7 proteins and by interacting with cellular proteins. In the following sections we will first describe the E6, E7 and E2 proteins in some detail. We will then outline how E2 influences the functions of E6 and E7, both by controlling the levels of these proteins and by altering their activity.

The E6 protein

The E6 proteins are around 160 amino acids in length and contain two zinc-binding motifs [88, 89]. The E6 proteins from high-risk HPV types are located in the nucleus and the cytoplasm, while the E6 proteins from low-risk HPV types are found predominantly in cytoplasm, due to the absence of two nuclear localisation signals (NLS) found in their high-risk counterparts [90]. Arguably the best understood function of the high-risk E6 protein is its ability to induce the degradation of p53 [34]. The E6 proteins from high-risk HPV types and low-risk HPV types bind to p53, but the interaction is weak in the case of the low risk proteins [91]. Furthermore, the high-risk and low-risk E6 proteins bind to different regions of p53. High-risk E6 proteins bind to the C-terminal region of p53 and the p53 core domain, whereas low-risk E6 proteins bind to the C-terminal region of p53 but not to the core domain [92]. Only the high-risk E6-p53 core domain interaction targets p53 for degradation [92].

p53 is involved in multiple processes including cell cycle regulation, the induction of apoptosis and DNA repair [93]. The half-life of p53 is very short, but in response to DNA damage or other cellular stresses p53 is stabilised and the protein functions as a DNA-binding transcription factor to induce the expression of the cyclin-dependent kinase inhibitor p21 and a variety of other target genes, resulting in cell cycle arrest and/or apoptosis. Activated p53 can also induce apoptosis by transcription-independent mechanisms that involve the translocation of p53 to the mitochondria and the release of cytochrome c [94, 95]. p53 can inhibit HPV DNA replication through a mechanism that appears to involve E2 [96–98]. In normal cells, the Mdm2 protein controls the levels of p53 by binding to p53 and acting as a ubiquitin ligase. Mdm2 relocates p53 from the nucleus to the cytoplasm for degradation by the proteasome. In turn p53 activates transcription of the Mdm2 gene, resulting in a control loop that keeps p53 levels low. The high-risk E6 proteins can overcome the cell cycle arrest and proapoptotic activities of p53 by targeting p53 for degradation and thereby inactivating the Mdm2 pathway [34, 99, 100]. Presumably, reducing the levels or activity of p53 also relieves the repression of HPV replication by p53.

High-risk E6 proteins bind to the p53 core domain in conjunction with E6-AP, a cellular E3 ligase that does not bind to p53 in the absence of E6 [36]. This leads to the ubquitination of p53 and its degradation by the 26S proteasome. Recent work has shown that high risk E6 proteins can also induce p53 degradation independently of E6-AP [100]. Furthermore, both high-risk and low-risk E6 proteins can inhibit the transcriptional activity of p53 independently of E6-AP by interacting with p300 and preventing the co-activation of p53 target genes [37]. These activities of E6 are summarised in Fig. 4. It is important to note that although cells expressing high-risk E6 proteins contain a reduced amount of p53, they can still respond to DNA damage and other signals that induce p53, and in some cases this leads to p53-induced apoptosis [101].

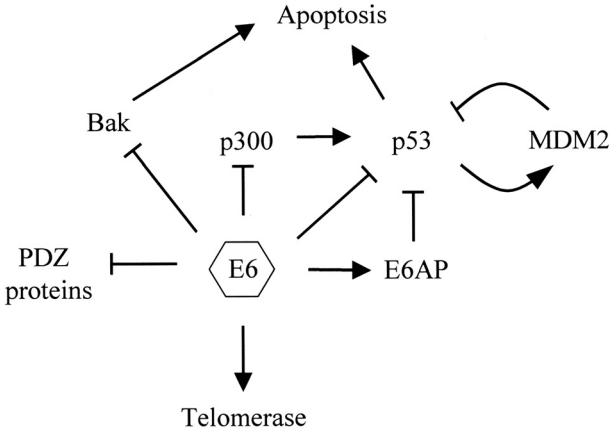

Figure 4. E6 targets multiple proteins. E6 controls p53 activity via several mechanisms. Down-regulation of p53 activity, activation of telomerase and the degradation of PDZ proteins by E6 promote tumourigenesis.

The high-risk E6 proteins target many other cellular proteins for degradation by the proteasome including the apoptotic effector protein Bak. Although the low-risk proteins also target Bak for degradation, they are less effective than the high-risk proteins [102]. The high-risk E6 proteins also interact with members of the PDZ family of proteins. The high-risk HPV E6 proteins bind specifically to PDZ domains and promote proteasome-mediated degradation of several family members including hDLG, MUPP-1, and hSCRIB [103–107]. In transgenic mice expressing the HPV 16 E6 protein, the ability to bind PDZ proteins appears to be required for the induction of cervical tumours [108, 109].

A function of the high-risk E6 proteins that is important for immortalization is their ability to activate the expression of the catalytic subunit of telomerase, hTERT [110]. Telomerase activity is usually found in embryonic cells and is absent in

somatic cells. As normal cells divide, the telomere regions shorten with each round of division, eventually producing chromosomal instability and senescence [111]. However, high-risk HPV E6 proteins have been shown to maintain telomeres through the action of telomerase [112–114]. Interestingly, over expression of hTERT in conjunction with E7 is sufficient to immortalize human primary keratinocytes [115]. The HPV E2 proteins are reported to repress hTERT promoter activity [116]. The interplay of E6 and E2 during the regulation of this promoter has not been investigated.

A considerable number of other cellular proteins have been reported to associate with high-risk E6 proteins including paxillin and p300/CBP [117–119]. Low-risk E6 proteins have also been documented to interact with several cellular proteins. However, a detailed description of these interactions is outside the scope of this work.

The E7 protein

The E7 proteins are around 100 amino acids in length and contain three conserved regions: CR1, CR2 and CR3 [120, 121]. Unlike the E7 proteins from low-risk HPV types, the E7 proteins from high-risk HPV types can immortalise primary human cells and can bring about cell transformation when expressed with an activated oncogene [122]. CR2 contains an LXCXE motif that mediates the binding of E7 proteins to members of the pocket protein family: pRb, p107 and p130 [38]. Rb plays key roles in DNA replication, DNA repair, the prevention of apoptosis, cell differentiation and cell senescence. The E7 proteins from high- and low-risk HPV types bind to Rb but the binding affinity of the high-risk E7 proteins for pRb appears to be around 10-fold higher than the low-risk E7 proteins [64]. This difference arises from a single amino acid difference in E7 which also determines the ability of E7 to cooperate with activated ras in transforming cells [123]. In its hypophosphorylated form, pRb controls transition at the G_1/S phase of the cell cycle by binding to the E2F family of transcription factors and repressing the transcription of E2F-target genes. In normal cells, pRb is hypophosphorylated in early G_1 and becomes increasingly phosphorylated by cyclin D/CDK4/6 complexes towards S phase. This results in the release of E2F proteins which then activate the transcription of genes required for S-phase transition. High-risk E7 proteins override this normal cell cycle control by binding to pRb and releasing E2F proteins [38]. High-risk E7 proteins also induce the degradation of pRb via a proteasome-dependent pathway, whereas low-risk E7 proteins do not target pRb for degradation [69, 124]. This suggests that the removal of pRb is important in tumourigenesis as well as its functional inactivation. One function of pRb is to repress transcription of the p16 cyclin-dependent kinase inhibitor and E7 therefore increases p16 levels. However, although p16 would normally inhibit CyclinD/CDK4 (and therefore pRb phosphorylation) to induce cell cycle arrest, this cannot happen since high-risk E7 proteins also remove pRb (see Fig. 5).

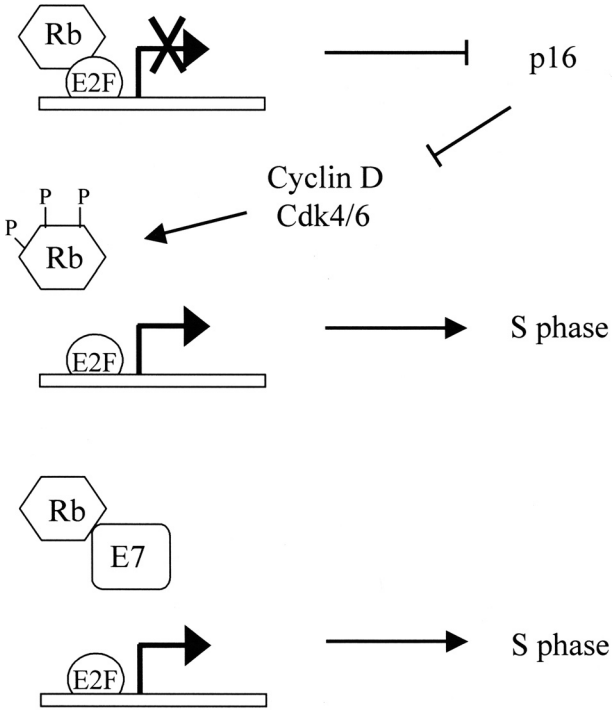

Figure 5. E7 targeting of pRb. Rb controls E2F activity and thereby regulates cell cycle progression. The binding of E7 to Rb releases E2F and thereby deregulates the cell cycle.

The p130 and p107 pocket proteins also target E2F proteins. Although pRb appears to function in the control of stem cell proliferation, p107 and p130 are involved in cell differentiation. Both low- and high-risk E7 proteins bind to p130 and target this protein for degradation [125]. This suggests that the deregulation of p130 is required to enable completion of the viral life cycle as infected cells migrate up through the epithelium. However, only high-risk E7 proteins target p107 for degradation, suggesting that the removal of this protein is not essential for completion of the viral life cycle [126].

It is important to point out that E7 can disturb normal cell cycle controls independently of these effects on the pocket proteins. Both high-risk and low-risk E7 proteins can inhibit cell senescence induced by the promyelocytic leukaemia protein (PML) [127]. Cell

senescence limits the replicative potential of cells and must be overcome during tumourigenesis. The E7 proteins bind to PML and block the ability of this protein to induce senescence. Mutations that block the binding of E7 to pRb bring about a slight reduction in the ability of E7 to block PML-induced senescence, suggesting that pRb-dependent and pRb-independent pathways are important for this function of E7 [127]. In addition, high-risk E7 proteins bind to E2F-1 leading to the activation of E2F-1-dependent transcription [128]. The affinity of E7 for E2F-1 appears to correlate with the oncogenic potential of the virus, in that high-risk E7 proteins are reported to have greater affinity for E2F-1 than low-risk E7 proteins [128]. E7 has also been shown to bind to cyclin-kinase complexes and cyclin-dependent kinase inhibitors. E7 binds directly to cyclin A/cdk2, as well as indirectly interacting with cyclin E/cdk2 complex via p107 [129, 130]. E7 can also bind to p21 and block p21-induced cell cycle arrest [131, 132]. E7 prevents p21 from inhibiting proliferating cell nuclear antigen (PCNA)-dependent replication and cyclin E/cdk2 activity and also from directly inhibiting E2F activity [131]. Both of these cyclin complexes can phosphorylate Rb and thus alleviate Rb-associated transcriptional repression.

High-risk and low-risk E7 proteins are associated with chromatin remodelling. By targeting a chromatin remodelling histone deacetylase complex, the E7 proteins can alter the chromatin structure of genes and therefore alter their expression. Histone deacetylases (HDAC) are expressed in all tissues and act to remove acetyl groups from the lysine-rich amino-terminal tail of the histone proteins. High-risk E7 proteins bind to HDAC-1 indirectly through Mi2β, a component of a nucleosome remodelling deacetylation complex [133]. E7 mutants that fail to bind Mi2β but which bind to Rb normally, fail to overcome cell cycle arrest.

In contrast to its pro-proliferative actions, E7 can stabilize p53 and induce apoptosis [134–136]. In targeting Rb, E7 causes deregulated E2F activity and E2F-1 can induce apoptosis in the presence of p53 [137]. E2F-1 activates expression of $p19^{ARF}$ which inhibits Mdm2 and thereby stabilizes p53 [138, 139]. However, E7-induced apoptosis can also occur in $p19^{ARF}$-deficient cell lines, indicating that other pathways must also exist [140]. Since it is thought that E7 can stabilize p53 by blocking its interaction with Mdm2, this demonstrates how important it is to consider E6 and E7 acting in the same cell at the same time.

The interplay of E6 and E7

In the vast majority of cervical carcinoma cells, the E6 and E7 proteins are continuously expressed and this is required for transformation. Prior to viral integration these proteins are believed to cooperate in order to overcome normal cell cycle controls. Expression of the E6 and E7 proteins from high-risk but not from low-risk HPV types brings about cell immortalization [41, 141, 142]. The E6 and E7 proteins of HPV 16 have been shown to cooperate to immortalize primary human keratinocytes and human epithelial cells [41, 141–143]. However, the E6 protein alone is unable to immortalize primary keratinocytes [141]. Although low-risk E6 and E7 proteins do not immortalize cells, low-risk E6 and high-risk E7 or high-risk E6 and low-risk E7 can cooperate to bring about immortalization [143]. Work using transgenic mice has shown that expression of either high-risk E6 or high-risk E7 alone is sufficient to induce hyperplasia and tumours. However, E7 alone induces many and mainly benign and differentiated tumours, whereas E6 alone induces few tumours but they are more malignant. This suggests that E6 promotes tumour progression [144, 145]. Malignant tumours occur at very high frequency in mice expressing both proteins [145]. High-risk E6 and E7 proteins cooperate to induce abnormal centrosome numbers, aberrant mitotic spindle pole formation, and genomic instability [146, 147].

The E2 Protein

E2 is approximately 360 amino acids in length and contains three functional domains (Fig. 6A and 6B); a carboxyl terminal DNA binding domain (DBD), an amino terminal transcription activation/DNA replication domain (TAD) and a central region thought to form a flexible hinge [148]. Molecular models of both the N- and C-terminal domains have been produced based on the results of X-ray crystallography and NMR studies. The DBD forms a dimer in which amino acids from both subunits create a β-barrel with four surface α-helices, two of which interact with DNA [149, 150]. The E2 DBD binds to DNA sequences that conform to the consensus sequence 5' aACCG N_4 CGGTt 3' where N represents any base pair [151, 152]. Crystal structures of the DBD in complex with E2 binding sites have been determined [149, 153, 154]. The N-terminal domain consists of glutamine-rich α-helices and β-sheets [155, 156] and also appears to be capable of dimerisation. However, due to the interdigitated nature of the β-barrel, the DBD appears to be an obligate dimer, whereas the interaction between individual N-terminal domains is weak and would be

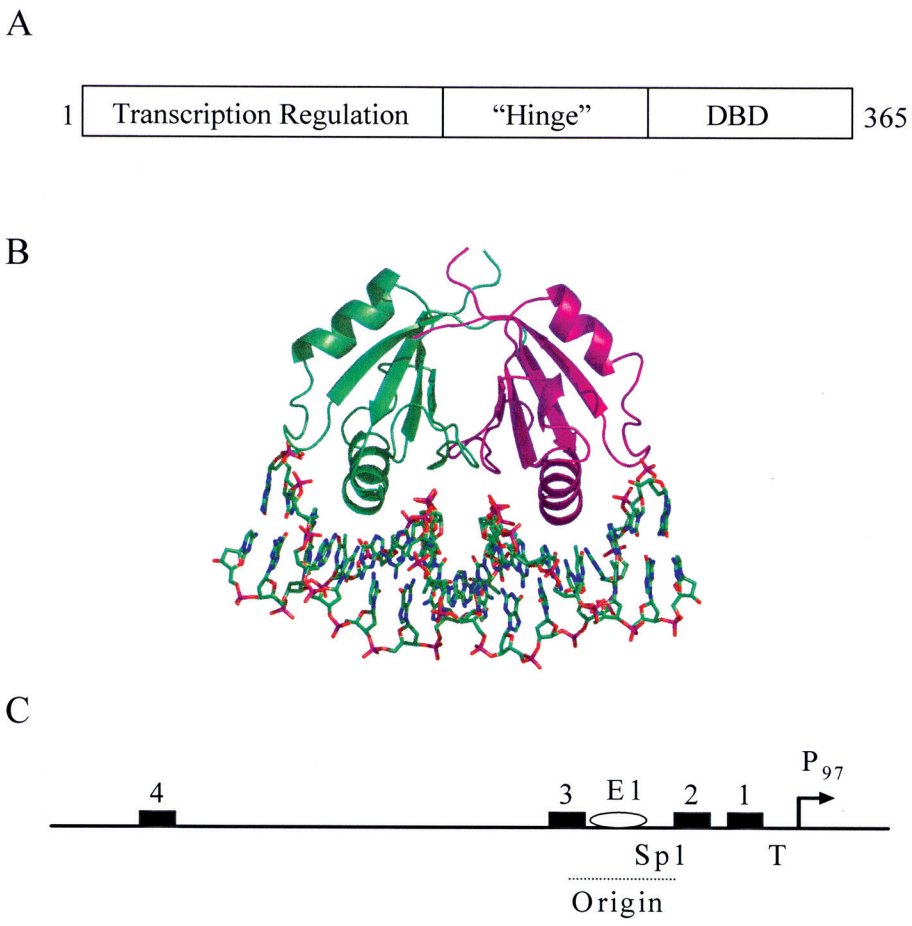

Figure 6. The E2 protein and the viral upstream regulatory region (URR). *(A)* The domain structure of the E2 protein. *(B)* The HPV 6 E2 DNA binding domain-DNA complex. The hinge region and transcription activation domain are not present. *(C)* The E2 and E1 binding sites within the HPV 16 URR are shown as filled boxes and an oval, respectively. The p97 transcription start site is indicated by the arrow. T and Sp1 indicate the TATA box and Sp1 binding site, respectively.

unlikely to occur if the DBD did not bring these domains into close proximity. The N-terminal domain is responsible for interactions with E1 and with host-cell transcription factors, including Brd4, Sp1, TFIIB and AMF-1, and is important for the transcriptional control of E6 and E7 [31, 157–159]. The hinge region links these two domains and is important in several aspects of E2 function, including protein stability [160] and protein localisation [161].

The regulation of HPV replication and transcription by E2

E2 is required for efficient HPV replication and is capable of transcription activation and repression [162–164]. There are four E2 binding sites within the HPV LCR (Fig. 6C). The role of E2 in HPV replication is relatively well understood. The binding of E2 to its promoter-proximal sites recruits E1 to the viral origin of replication [23]. Once E1 is bound, E2 is displaced from the origin and replication can begin [25]. In contrast, the role of E2 in the control of HPV gene expression is less well understood, although a variety of models have been suggested to describe how E2 might regulate HPV gene expression during the viral life cycle. When E2 binding sites are placed upstream of a minimal promoter, the E2 proteins can activate transcription [164]. This suggests that the binding of E2 to the promoter-distal sites within the LCR could activate HPV transcription. However, when HPV E2 proteins bind to the LCR they generally bring about the repression of promoter activity. The mechanism by which repression is brought about has been elucidated in detail. Two promoter-proximal E2 binding sites are flanked by an Sp1-binding site and a TATA box sequence. When E2 binds to the promoter-proximal E2 sites, it brings about the displacement of Sp1 and TFIID leading to repressed transcription [165]. In addition to competing with Sp1 and the TATA box binding protein (TBP) for binding to DNA, E2 can also interact with both of these proteins [166, 167]. It has been proposed that at early stages of infection low levels of E2 might favour the binding of E2 to a high affinity promoter-distal E2 binding site, resulting in transcription activation; whereas at later times of infection and higher E2 protein levels, E2 might bind to the promoter-prox-

imal E2 sites leading to the repression of transcription. However, the HPV 6 E2 protein binds tightly to a promoter-proximal E2 site in the HPV 6 genome and the HPV 11 E2 protein binds equally well to all of its sites within the HPV 11 genome [168, 169]. This suggests that different HPV types might have different mechanisms of transcription regulation.

There is relatively little data on the regulation of LCR activity by E2 during a viral infection. Mutation of the promoter-proximal E2 binding site appears to relieve E2-mediated transcriptional repression in the context of a whole HPV 31 genome [170] and the HPV 16 E2 protein represses transcription from the HPV 16 LCR in an HPV-Epstein-Barr virus (EBV) model system that maintains the LCR in an episomal replicon [171]. In addition, the transcription activation function of the HPV 31 E2 protein is not required for the viral life cycle [172], suggesting that the transcriptional repression function of E2 may be all that is required or that only the replication function of E2 is important. Importantly, the HPV 16 E2 protein does not repress HPV 16 transcription when the LCR is contained within an episomal HPV genome [173]. To complicate matters further, alternative splicing produces a protein known as E8E2C that consists of a short open reading frame fused to the E2 DNA binding domain [174]. The E8E2C protein is a transcriptional repressor protein that appears to be required for extrachromosomal maintenance of some but not all HPV types [174–176]. The interplay between E2 and E8E2C in transcriptional regulation during viral infection is not well understood.

The E2 proteins interact with a variety of viral and cellular proteins that can modulate its replication and transcriptional activities. As mentioned earlier, E2 binds to the HPV E1, L2, E6 and E7 proteins and the cellular proteins TBP, Sp1 and p53. The binding of p53 to E2 inhibits HPV DNA replication and can alter the transcriptional activity of E2 [97, 98]. Similarly E2 interacts with topoisomerase II binding protein 1 (TopBP1) and this has consequences for replication and transcriptional regulation [177]. It would seem likely that these and other E2-interacting proteins modulate HPV replication and transcription during viral infection. Similarly, E2 can modulate the activity of many cellular proteins. A particularly important example being the APC activators Cdh1 and Cdc20 through which the high-risk E2 proteins might induce genomic instability [178]. However, as yet very few experiments have looked at the importance of these interactions in systems that recapitulate the HPV life cycle.

The effects of E2 on HPV-transformed cells

As described above, disruption of the E2 or E1 genes is a common event in cervical tumours resulting in the loss of E2 and the subsequent deregulation of E6 and E7. Reintroduction of E2 into cervical carcinoma cells has been shown to repress transcription of the integrated E6 and E7 genes and induce growth arrest, apoptosis and cellular senescence [72, 179–181]. As might be expected, the precise outcome of E2 reintroduction appears to depend on the virus type and the system used to express the protein. The introduction of bovine papillomavirus (BPV) E2 causes growth arrest in HPV-transformed HeLa and SiHa carcinoma cells, but not in the HPV-negative cell line C33A [73, 82, 181, 182]. The introduced E2 represses transcription from the p105 (HPV 18) and p97 (HPV 16) promoters respectively, which regulate the expression of E6 and E7 [28, 73]. Repression of these promoters restores the normal functions of p53 and pRb, as well as other targets of E6 and E7 (Fig. 7) [72, 183–188]. Reintroduction of E2 in combination with either E6 or E7 under the control of an E2-independent promoter has been used to further elucidate the functions of E6 and E7 in transformed cells [72, 82, 185, 189]. Repression of E7 alone activates the pRb pathway but not p53 pathway and triggers cell senescence [190]. On the other hand, repression of E6 alone results in the activation of p53 and the induction of senescence and apoptosis [191]. The BPV E2 protein and the E2 proteins from HPV 16 and 18 have all been shown to induce apoptosis in cervical carcinoma cell lines [73, 181, 192, 193]. Apoptosis appears to be an early consequence of E2 expression occurring within a few hours or days and it may be that cells which survive this initial wave of apoptosis go on to exhibit cell senescence and growth arrest. The high-risk E2 proteins are highly efficient inducers of apoptosis and this has lead to the suggestion that these proteins could be used in cancer therapy [194–196]. The low-risk HPV 6 and HPV 11 E2 proteins do not induce apoptosis [74, 197]. This suggests that the ability to induce apoptosis might be related in some way to the loss of E2 that occurs during tumourigenesis. It appears that the E2 proteins which induce apoptosis do so via multiple pathways. BPV E2- and HPV 18 E2-induced apoptosis occurs in the absence of an increase in *Bax*, one of the p53 target genes that can induce apoptosis [198]. This suggests that E2-induced apoptosis occurs via a p53-independent pathway. In agreement with this conclusion, E2 can induce apoptosis in p53-null cells, at least when the protein is expressed at high levels [199]. This p53-independent apoptosis involves the activation of caspase 8 triggering the extrinsic apoptosis pathway.

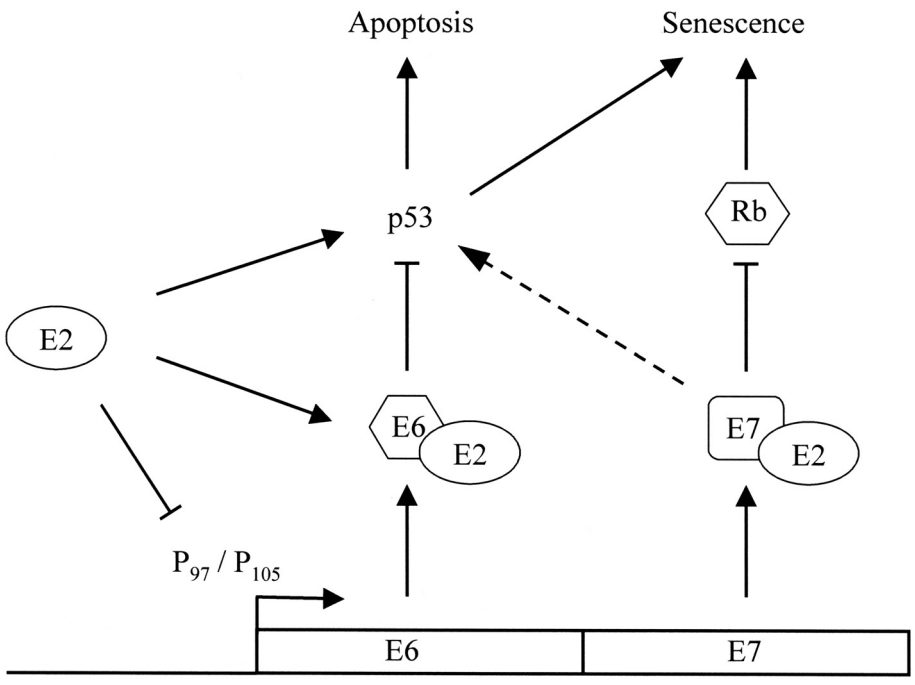

Figure 7. E2 influences apoptosis and cell senescence via multiple pathways. E2 represses HPV gene expression and thereby indirectly modulates apoptosis and cell senescence via E6 and E7 expression levels. E2 can also interact directly with E6, E7 and p53 to influence apoptosis and senescence.

The high-risk E2 proteins but not low-risk E2 proteins activate caspase 8 and are themselves substrates for this protease [197, 199]. However, the high-risk HPV E2 proteins can also induce apoptosis via p53-dependent mechanisms. At low expression levels, the HPV 16 E2 protein does not induce apoptosis in p53-null Saos-2 cells, unless the protein is co-expressed with p53 [192]. Furthermore, mutations in E2 that block the E2 DBD-p53 interaction block the induction of apoptosis by E2 in these cells. Further evidence to suggest that E2 can induce apoptosis via a p53-dependent pathway comes from the fact that in some experiments E2-induced apoptosis can be blocked by over-expression of the HPV 16 E6 protein [192]. The situation is complicated by the fact that p53 can induce apoptosis by transcription-dependent and transcription-independent pathways. As mentioned previously, some inducers of p53 trigger an initial apoptotic response that does not require the activation of transcription. It would seem likely that high risk HPV E2 proteins at least trigger apoptosis via this p53 transcription-independent pathway, by an unrelated p53-independent pathway and later, by the p53 transcription-dependent pathway. In non-HPV-transformed normal human keratinocytes, the HPV 31 E2 protein has been shown to reduce p53 levels [200]. The mechanism by which this E2 protein brings about a reduction in p53 levels is not known. However, this observation suggests that the E2 proteins can have a direct effect on the level of p53 as well as an indirect effect via the regulation of E6 expression.

E2 in viral infection and tumourigenesis.

The repressive effects of E2 on the proliferation of HPV-transformed cells imply that E2 might also influence cell proliferation during the normal HPV life cycle. As infected cells rise up through the epithelium, they undergo terminal differentiation, a process of programmed cell death with several features in common with apoptosis. It is possible that E2 is involved in delaying terminal differentiation at the early stages of infection and/or inducing terminal differentiation at late stages of infection. It is also possible that at early stages of infection E2 might induce true apoptosis. This could facilitate the spread of HPV genomes between neighbouring cells via the phagocytosis of apoptotic bodies containing viral DNA [201]. However, there is no evidence to suggest that there is significant apoptosis in HPV-induced lesions [202]. It would seem more likely then that rather than inducing apoptosis or growth arrest, E2 acts in conjunction with E6, E7 and possibly other viral proteins to facilitate the HPV life cycle.

E2 could affect the survival of infected cells by at least two mechanisms; indirectly via the regulation of E6 and E7 expression or directly via binding to viral and cellular proteins and possibly cellular DNA sequences. The indirect and direct effects of E2 can operate in HPV-infected cells but will generally be lost in HPV-transformed cells due to the frequent loss of E2. As described above, very few experiments have addressed the role of E2 in the control of E6 and E7 transcription during viral infection. Changes in E2

protein levels, HPV genome copy number and changes in the levels and activities of the E6 and E7 proteins will all affect how E2 controls transcription of E6 and E7 and have consequences for cell proliferation. However, partly due to the low expression levels of these proteins (at least during the initial stages of the life cycle), and partly due to the lack of reliable antibodies, we do not have a clear picture of how the relative levels of these proteins alter during infection. E2 levels appear to increase as infected cells migrate to the surface of the epithelium [203]. E6 and E7 levels are also likely to increase. However, a detailed comparison of protein levels and HPV DNA levels has not been made.

To complicate matters further, E6 binds to E2 across the E2 DBD [75]. The binding of E2 to E6 inhibits the degradation of PDZ proteins by E6, but has little effect on the degradation of p53 [75]. This might help or allow infected cells to escape the repression of HPV replication by p53. The binding of E6 to E2 also alters the localization of E2, resulting in its accumulation in nuclear speckles, and increases the transcription activation function of E2 at a synthetic reporter promoter [75]. These findings lead to the suggestion that the loss of E2 results in increased E6 activity and that this might favour malignant progression [75]. However, the effect of this interaction on LCR activity has not yet been reported. In addition, E7 can bind to the hinge region of E2 [76]. The binding of E2 to E7 increases the stability of E7 but inhibits the transformation potential of E7 delivered in conjunction with activated ras [76]. This suggests that the loss of E2 might result in increased transformation by E7 [76]. We do not know whether the interaction between E2 and E7 alters the ability of E2 to regulate LCR activity. Similarly, we do not know whether E6 and E7 can bind simultaneously to E2, or whether these interactions separate E2 into two populations. It will be interesting to determine whether low-risk E6 and E7 proteins bind to low-risk E2 proteins. Presumably they do but loss of the interactions, whilst releasing E6 and E7 from constraint by E2, would not transform cells since these proteins have greatly reduced transforming activity. Obviously the reintroduction of E2 into HPV-transformed cells would reinstate these E2-E6 and E2-E7 interactions.

Although p53 levels are reduced in cells expressing E6, the E2 protein could also influence cell proliferation via its interaction with this protein. Certainly in the absence of E6, the HPV 31 E2 protein can down-regulate p53 levels and induce apoptosis [200]. The HPV 16 E2 protein can also induce apoptosis in the absence of E6 and other viral proteins and this again appears to operate via p53 [192]. Similarly, E2 could influence cell proliferation via several other cellular proteins. It is accepted that integrated HPV genomes give cells a selective advantage over cells with episomal HPV genomes due to the up-regulation of E6 and E7 expression. As well as allowing this up-regulation of oncogene expression, the loss of E2 might remove constraints on cell proliferation adding to this selective advantage. Increased host genome instability caused by the binding of high-risk E2 proteins to Cdh1 and Cdc20 might drive HPV genome integration [178]. In this regard it is also worth remembering that integrated and episomal genomes probably coexist for long periods of time during high-risk infections. The continued expression of E2 from the episomal genomes might contribute to the emergence of cells with solely integrated genomes. The immune system may also come into play at this stage, since E2 is immunogenic and cells expressing E2 may be more likely to be targeted for elimination by the immune system [204, 205].

Finally, although the extent to which E2 directly regulates the expression of cellular genes is in debate, it is also likely that E2 influences cell proliferation by regulating the expression or activity of key cellular proteins. Interestingly, expression of the HPV 8 E2 protein in transgenic mice in the absence of other HPV proteins can induce the formation of skin tumours [206]. The mechanism by which this E2 protein acts as an oncogene is not known. Other E2 proteins have been reported to repress the activity of the hTERT promoter [183], although this would be expected to inhibit cell proliferation. It is likely that further cellular targets of the E2 proteins remain to be identified.

Conclusions

Completion of the viral life cycle requires the coordinate action of the HPV proteins. It would seem likely this is brought about by regulated gene expression and, at least in part, by interactions between the viral proteins. The consequences of the physical and genetic interactions between E2 and its various partner proteins during the viral life cycle are hard to predict. However it would seem likely that in viral infections E2 is subject to regulation by E6 and E7 and vice versa. This could result in the operation of feedback loops that control not only the expression of E6 and E7, but also their effects on cell proliferation and cell survival. The effects of individual viral proteins on each other are relatively easy to investigate. However, the complex interplay between multiple proteins is difficult to address. For example, although we know that E2 has the potential to interact with E6 and E7, we do not know how these three

proteins will interact during the course of a viral infection as the levels and activities of each protein change. It is clear that cellular proteins also interact with multiple HPV proteins and that these interactions are likely to be even more complex. p53, for example, binds to E2 and E6 as well as to many cellular proteins. Understanding how these complex sets of interactions are exploited by the virus will require experimental approaches that do more than simply study the individual components.

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